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Contents

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VOL. 25, SEC. E. AUGUST, 1947 NUMBER 4

THE ESTIMATION OF TRYPSIN[1]

BY F. D. WHITE[2] AND J. M. BOWMAN[3]

Abstract

Details are given of a colorimetric method for the estimation of trypsin that can be utilized for the evaluation of the proteolytic activity of pancreatic secretion. Being an adaptation of a method that has been used successfully in a clinical laboratory for many years, it is suitable for routine clinical use. Like Anson's method it depends upon the colour reaction between the tyrosine–tryptophane radicals of a protein substrate and Folin's phenol reagent, but embodies the following features: the results cannot be influenced by the presence of peptidases in the enzyme solution, since the procedure is based upon the estimation of a casein substrate before and after digestion, and not upon the digestion products; the digestion is carried out at 37° C.; by reference to a curve the enzyme content can be expressed in terms of trypsin nitrogen. Results obtained with this method showed a reasonable degree of constancy, the average deviation from the mean being ±3.3% and the maximum deviation, ±8.4%.

Introduction

The proteolytic activity of pancreatic secretion, when poured into the duodenum, exercises a profound effect upon the process of digestion, and consequently the evaluation of the protease content of this secretion, in health and disease, is a matter of considerable importance. This activity is due to the presence of at least two enzymes, trypsin and chymotrypsin, but as their digestive action upon the protein molecule is similar, that of trypsin is generally taken as typical of the proteolytic activity of the secretion. Several methods of estimation have been suggested by different investigators but no single one has been adopted generally, and this investigation was begun with the object of ascertaining which of these procedures was best suited for adoption as a routine method for clinical use.

The digestion of protein by pancreatic secretion consists of a series of reactions whereby the complex molecule is hydrolysed to a number of simpler molecules, with a consequent increase in the number of carboxyl and amino groups, the first detectable chemical change being the conversion of the protein into a form no longer precipitable by trichloracetic acid. Of the chemical methods for the evaluation of proteolytic activity those mostly used are the estimation of the "non-protein nitrogen" (i.e. nitrogen remaining in solution after precipitation with trichloracetic acid) by micro-Kjeldahl, and

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the formol titration method in which the increase in the number of carboxyl groups is measured. (Cf. Northrop and Kunitz (9).) Neither of these methods is especially suitable for clinical practice since they both depend upon titration procedures in which the significant differences are small.

A more suitable method has been proposed by Anson (2), in which after trichloracetic acid precipitation, the filtrate containing the soluble products of digestion is treated with the Folin phenol reagent (4) in presence of alkali and the colour produced compared against a standard solution of tyrosine, treated similarly. This method however is not ideal. In common with all other procedures investigated, the estimation is carried out on the products of digestion, and since pancreatic secretion also contains peptidases, the end products of digestion are not necessarily a definite measure of the amount of protease present. Again as in other methods, the proteolytic activity is expressed in arbitrary units. As a routine procedure it has the disadvantages that the digestion is carried out at 25° C., not a common practice in a clinical laboratory, and since the estimation depends upon the direct measurement of colour, the slight colour produced by the reagents alone is of significance and makes a blank estimation essential.

In an attempt to overcome these objections, attention was directed to the possibility of estimating the protein before and after digestion, and thus determining the amount of digestion by difference. In 1936, Cameron, Guthrie, and White (3) published a method for the estimation of the proteins of blood plasma that is essentially similar to Anson's method except that the colour is produced by the reaction of the phenol reagent with the precipitated protein, instead of with the filtrate. Since this method has been in use in the Biochemistry Laboratory of the Winnipeg General Hospital for over 10 years and has proved satisfactory, it was taken as the basis of the procedure for the estimation of trypsin now reported, which can be considered as a modification of Anson's method designed for routine use in a clinical laboratory.

Any method based on the colour produced by the interaction of the phenol reagent with protein or protein products depends upon the tyrosine and tryptophane content of the protein. In much of the work reported in the literature casein prepared "according to Hammarsten" was the substrate of choice, but owing to doubts as to the homogeneity of this preparation, Anson replaced it with haemoglobin. We have reverted to the use of casein, the difficulty of the variation in tyrosine–tryptophane content of different batches being overcome by determining the "tyrosine factor", i.e. the ratio of the protein nitrogen in unit quantity to the chromogenic value, expressed as tyrosine, of the same quantity. Use of this factor enabled the amount of digestion to be expressed in terms of casein nitrogen.

All digestions were carried out in a water-bath fitted with a thermostat at 37° C., $\pm$ 0.1°. This temperature was chosen since in most laboratories an incubator or oven is kept permanently at 37° C.

To determine the suitability of the method, curves were plotted from the results of digestion experiments with both crude trypsin and the pure crystalline

enzyme, and in accordance with Northrop's observation (6), the former was found to be almost that of a monomolecular reaction, while with pure trypsin the velocity constant progressively decreased. From these results a curve was constructed relating the amount of digestion to the concentration of trypsin, and experiments carried out to determine the degree of error involved in evaluating digestion by this method. Details are as follows.

Method

Preparation of Substrate

Add 15 gm. of casein "according to Hammarsten" to 125 cc. of distilled water containing 4 mgm. of merthiolate per 100 cc., allow to stand with occasional shaking for several hours, and then add 35 cc. of 0.2 *N* sodium hydroxide slowly and with continuous agitation. When the casein has almost completely dissolved add 90 cc. of *M*/15 phosphate buffer, pH 7.4, and place in the refrigerator for 24 hr. At the temperature of the refrigerator this solution will keep for several weeks. For the estimation, dilute one volume of this solution with four volumes of the *M*/15 phosphate buffer. The final pH is 7.5.

Procedure

Pipette 20 cc. of the diluted substrate into each of two test tubes, one of which is placed in a water-bath or incubator kept at a constant temperature at 37° C. To the other add 1 cc. of the enzyme solution under examination, mix by inversion, and *immediately* transfer by pipette 1 cc. to a centrifuge tube containing 2 cc. of water. Add 1 cc. of 20% trichloracetic acid and stir well with a fine glass rod.

To the test tube in the water-bath, the contents of which should be at 37° C., also add 1 cc. of the enzyme solution, mix, and allow digestion to proceed for exactly 15 min. At the end of this time remove 1 cc. by pipette and transfer it to a second centrifuge tube containing 2 cc. of water. Add 1 cc. of 20% trichloracetic acid and stir as before.

After letting the tubes stand for at least five minutes, remove the rods from both, washing them down with a cc. or two of 5% trichloracetic acid, and centrifuge. Pour off and discard the supernatant liquid, add to each tube 5 cc. of 5% trichloracetic acid, and stir thoroughly. Remove and wash the rods and again centrifuge. Pour off the supernatant liquid, invert the tubes, and drain over filter paper.

To the precipitated protein in each tube add 1 cc. of 10% sodium hydroxide; place the tubes in a boiling water-bath, small filter funnels being inserted in the mouths of the tubes to lessen evaporation. After 30 min. remove the tubes, dilute with 7 cc. of water, add 1 cc. of the phenol reagent, and 3 cc. of 20% sodium carbonate. Mix by inversion, allow to stand for 15 min., and compare in the colorimeter against a standard solution of tyrosine, set at 10 mm.

The Tyrosine Standard

Dissolve 200 mgm. of pure tyrosine in 1 litre of 0.1 *N* hydrochloric acid. For the estimation take 2 cc. of this solution (= 0.4 mgm.) and add 1 cc. of 10% sodium hydroxide, 5 cc. of water, 1 cc. of phenol reagent, and 3 cc. of 20% sodium carbonate, in this order. Mix and let stand for 15 min.

Calculation

Cameron, Guthrie, and White found that in the case of plasma proteins, to get true proportionality between colour development and concentration of protein, it was necessary to apply the correction, $y = 1.1x - 1$, where x is the reading in mm. of the protein solution with the standard set at 10 mm., and y is the true reading. This has also been found true where the protein is casein, and consequently all colorimetric readings are corrected accordingly. The concentration of protein in 1 cc. of the substrate is therefore equivalent to $\frac{0.4 \times 10}{y}$ mgm. of tyrosine, whence the amount of casein nitrogen in the substrate is given by the equation:—

$$\frac{0.4 \times 10 \times F \times 21}{y} \text{ or } \frac{84 \times F}{y} = \text{mgm. casein nitrogen}$$

where F is the tyrosine factor.

The difference between the value found for the control and that of the digested specimen gives the amount of digestion, in terms of casein nitrogen, produced by 1 cc. of the enzyme solution. By reference to the curve (Fig. 3) this can be converted into μgm. trypsin nitrogen.

Note on the Phenol Reagent

The phenol reagent referred to is that of Folin and Ciocalteu, which differs from that of Wu (10) by the addition of lithium sulphate. Cameron *et al.* used the Wu reagent and it can also be used for this procedure if the colour is allowed to develop for 30 min. instead of 15, and if the tubes are then centrifuged to remove the precipitate that forms. The Folin reagent is more convenient and gives the same results.

Experimental Part

The Tyrosine Factor

This was obtained by determining by the method described, the chromogenic value in terms of tyrosine of 1 cc. of the diluted substrate, and comparing it with the protein nitrogen of the same solution as determined by the Kjeldahl procedure.

On four separate samples the colorimetric reading gave a mean value of 5.93 mm. (corr.) and hence was equivalent to 0.674 mgm. of tyrosine.

Four Kjeldahl estimations gave a mean value of 1.48 mgm. of protein nitrogen per cc.

Hence the tyrosine factor was $\frac{1.48}{0.674} = 2.2$.

Most of the experimental work was carried out with casein having this tyrosine factor, but for some of the work a batch of casein obtained from a different source was used, and it was found to have a factor of 2.0. Since it is obviously essential to determine this factor for each fresh batch of casein, it is recommended that an amount sufficient to last for a year or two be collected, well mixed, and the factor determined.

Construction of the Activity Curve

The trypsin used in these experiments was an active commercial preparation, a light-coloured apparently homogeneous powder, the actual enzyme content of which was unknown. Solutions were made varying in strength from 1 to 10 mgm. per cc. In the first series of experiments, 1 cc. of the enzyme solution was added to 20 cc. of the substrate, 1 cc. quantities withdrawn at different time intervals and the amount of digestion determined as described and plotted against time of digestion. The curves obtained, of which the upper curve in Fig. 1 is typical, showed that in these experiments the rate of digestion was approximately proportional to the time for the first 20 min., after which there was a gradual decrease. This led to the adoption of a 15 min. period

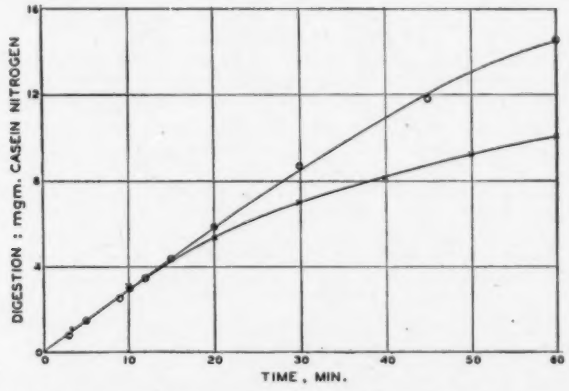

FIG. 1. *Curves showing rate of digestion of casein substrate at 37° C., with crude and crystalline trypsin: circles represent values for crude trypsin; crosses, those for crystalline trypsin.*

for the second series, in which the enzyme concentration was varied. The upper curve in Fig. 2 shows the relationship between amount of digestion and concentration of enzyme. To correlate this with actual trypsin content the pure enzyme was prepared in crystalline form according to the method of Northrop (7). A suitable amount was dissolved in water, the concentration of the solution being determined by reprecipitation with 2.5% trichloracetic acid, and nitrogen estimated by Kjeldahl on the precipitate. The digestive activity was determined (*a*) at intervals up to 60 min., and (*b*) with varying concentrations after 15 min. digestion. The resultant curves are shown in Figs. 2 and 3.

Northrop (6, 8, 9) has shown that digestion is only proportional to enzyme concentration in the early stages of the reaction and that therefore only the initial slope of the curve can be used as a basis of evaluation. Accepting that, in conjunction with the fact that for any stated amount of digestion, the

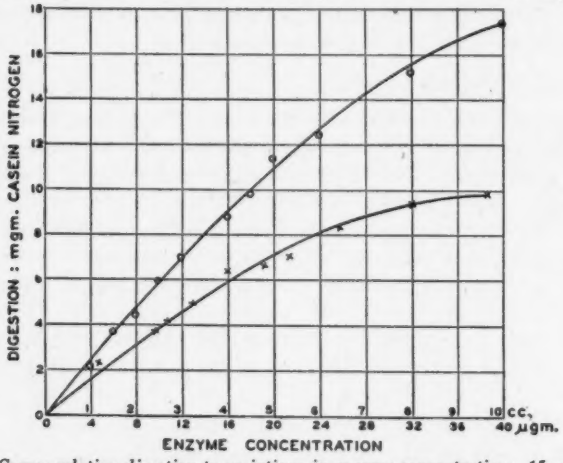

FIG. 2. *Curves relating digestion to variations in enzyme concentration: 15 min. digestion at 37° C.; values for crude trypsin, in cc., represented by circles; concentration of crystalline trypsin expressed in terms of µgm. trypsin nitrogen and represented by crosses.*

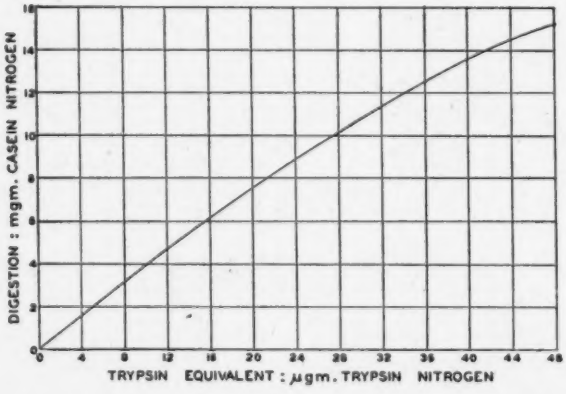

FIG. 3. *Activity curve. Relation of proteolytic activity, represented by digestion of 20 cc. of casein substrate for 15 min., to corresponding amount of trypsin, expressed as µgm. trypsin nitrogen digesting at an initial rate of 1 mgm. casein nitrogen per minute.*

concentration of enzyme will be inversely proportional to the time of digestion, the activity curve was constructed as follows:—

The initial slope of the curve for crude trypsin (cf. Fig. 2) was produced until it intersected the horizontal line representing a digestion of 5 mgm.

casein nitrogen in the 15 min. period. This point corresponded to 2.1 cc. of enzyme solution. Multiplying this by 15 and dividing by 5 gave 6.3 cc. the amount of solution that, acting for one minute, would give a digestion of 1 mgm. casein nitrogen. Applying the same procedure to the curve for crystalline trypsin, the corresponding figure was 12.6 μgm. trypsin nitrogen, whence 1 mgm. casein nitrogen would be digested in one minute by 12.6 × 3, or 37.8 μgm. trypsin nitrogen. Therefore at the initial rate of digestion of 1 mgm. casein nitrogen per minute, 1 cc. of the enzyme solution would digest the same amount as 37.8/6.3 or 6.0 μgm. of trypsin nitrogen. The crude trypsin curve was then replotted to give the amount of digestion against the corresponding amounts of trypsin nitrogen, and is shown in Fig. 3.

This is an attempt to express the proteolytic activity of pancreatic secretion in terms of trypsin rather than in units. Since the crude trypsin probably, and pancreatic secretion certainly, owes its proteolytic activity to chymotrypsin as well as to trypsin, it is obviously impossible to determine from that activity the actual amount of trypsin involved. The points on the curve therefore represent the amount of enzyme that, acting on 20 cc. of substrate for 15 min., produces an amount of digestion equivalent to that produced by the corresponding amount of trypsin digesting at an initial rate of 1 mgm. casein nitrogen per minute.

The Average Degree of Error

For the method to be of use for the evaluation of proteolytic activity it must give results independent of enzyme concentration, and these results must show a reasonable degree of constancy. Table I shows the results obtained in a series of determinations with three samples of different commercial preparations of crude trypsin and two samples of duodenal contents. In the case of the crude trypsin a solution was made in which the enzyme content was designated as 1. From this, by a series of dilutions, solutions were obtained in which the enzyme content bore a fixed ratio to that of the original solution. The samples of duodenal contents were treated similarly. Digestion of the substrate was carried out with 1 cc. of each solution. From the results obtained the enzyme content in terms of trypsin nitrogen of each solution was read off from the graph, and the content of the original solution calculated. The results of 21 separate determinations show an over-all average deviation from the mean of ±3.3%, with a maximum deviation of ±8.4%.

Comparison with Anson's Method

Anson used as substrate a 2.2% solution of haemoglobin, to 5 cc. of which the enzyme was added and digestion carried out for 10 min. at 25° C. The total mixture was then precipitated by trichloracetic acid. We have used as substrate 20 cc. of casein solution containing about double the amount of protein, the digestion was at 37° C. for 15 min., and then only 1 cc. of the mixture was precipitated. Direct comparison of the two methods was therefore difficult, but an approximation was achieved by carrying out a

TABLE I

EVALUATION OF PROTEOLYTIC ACTIVITY IN TERMS OF TRYPSIN

Solution	Relative amt. of enzyme per cc.	Amount casein digested, mgm. nitrogen	Enzyme content, expressed as trypsin nitrogen			Average deviation from mean		Maximum deviation from mean,
			From graph, μgm.	Per cc. solution, μgm.	Mean value, μgm.	μgm.	%	%
Crude trypsin *A*	1	8.62	23.2	23.20				
	0.8	6.64	17.4	21.75				
	0.6	5.30	13.6	22.70				
	0.4	3.15	8.0	20.00				
	0.2	1.71	4.3	21.50	21.83	±0.90	±4.1	±8.4
B	1	15.16	47.5	47.50				
	0.8	12.60	36.4	45.50				
	0.6	9.50	26.0	43.30				
	0.5	8.07	21.6	43.20				
	0.4	6.67	17.4	43.50				
	0.3	5.46	14.1	47.00				
	0.2	3.48	8.9	44.50	44.90	±1.49	±3.3	±5.8
C	1	9.87	27.0	27.00				
	0.75	7.36	19.5	26.00				
	0.5	4.93	12.7	25.40	26.13	±0.58	±2.2	±3.3
Duodenal contents *A*	1	10.50	29.2	29.20				
	0.8	9.12	24.7	30.87				
	0.6	6.60	17.3	28.83				
	0.4	4 80	12.3	30.75	29.91	±0.90	±3.0	±3.6
B	1	7.85	20.8	20.80				
	0.33	2.55	6.4	19.20	20.00	±0.80	±4.0	±4.0

Over-all average deviation from mean = ±3.3%

series of digestions with the casein substrate at 37° C., and at appropriate intervals estimating 1 cc. by our method and 5 cc. by Anson's technique, expressing the values from the latter in mgm. casein nitrogen (using the tyrosine factor) instead of in milliequivalents of tyrosine.

The curves representing the rate of digestion were substantially the same, but the amount of protein digested at the varying time intervals as determined by our method was consistently greater than that by Anson's method. Since the initial step is the same in both procedures, precipitation of undigested protein with trichloracetic acid, theoretically the chromogenic value of the filtrate (or centrifugate) should equal the diminution in chromogenic value, after digestion, of the protein. The reason for this discrepancy is not clear, but a possible explanation may lie in the heating with sodium hydroxide prior to colour development, which is part of our technique, whereas Anson's procedure is carried out at room temperature.

Andersch and Gibson (1) observed that a prior heating of the plasma proteins with alkali greatly intensified the degree of colour obtained with the

phenol reagent. This has been amply confirmed for plasma proteins (3, 5) and we found that casein was similarly affected. This can only mean that not all the tyrosine and tryptophane radicals in these proteins are free to react directly with the phenol reagent, since our experiments have shown that the chromogenic value of the free amino acids is unaffected by prior heating. Anson's procedure measures the products of enzymatic digestion, which presumably include proteoses and peptones soluble in trichloracetic acid, and it is a question whether the tyrosine–tryptophane radicals in these are all free to react. We have carried out a few experiments to test this possibility, and found that two out of three peptone preparations showed an increase in chromogenic value on heating. One of these, it was claimed, was a preparation especially purified for bacteriological use: it was easily soluble in water and gave no precipitate with trichloracetic acid. We intend to pursue this question further.

Acknowledgments

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THE VITAMIN A ACTIVITY AND CAROTENE CONTENT OF TOMATO JUICE[1]

By L. I. Pugsley,[2] G. Wills,[3] and T. D'Aoust[3]

Abstract

The vitamin A activity was determined biologically and carotene content colorimetrically on 14 different commercial brands of tomato juice and on the juice prepared from eight different varieties of tomatoes. The vitamin A activity and the carotene content of the commercial brands were well within the range of the values obtained for the different varieties and a probable correlation was found between the vitamin A activity and the carotene content of the different varieties. The mean value for the vitamin A activity of the commercial brands was 11.6 I.U. per cc. (329 I.U. per ounce) showing that tomato juice may contribute significantly to the dietary vitamin A. The amount of lycopene-like compounds in tomato juice is considerably higher than the carotene compounds and was found to vary between different brands and different varieties. A substance was shown to be present in processed tomato juice that had the properties of α-tocopherol in biological assays for vitamin A activity.

The vitamin A activity of tomato juice is usually expressed in terms of the carotene content. In view of the number of isomers of carotene present and the relatively high lycopene content of tomato products, difficulties have been encountered in obtaining a reliable evaluation of the vitamin A activity of this material. Oser and Melnick (6) found a good correlation between the bioassay estimates of vitamin A potency and those predicted from the results of colorimetric analyses of crude carotene for heat processed vegetables, including tomato products. Guerrant *et al.* (4) have shown that the retention of carotene during the canning of tomato juice is from 60 to 74%, which is considerably less than the percentage retained by canned tomatoes. Zcheile and Porter (8) made a spectroscopic study of the carotenoids in tomato products and compared this with the results obtained chromatographically. They consider that the carotene in these products is the best index of provitamin A activity, but other carotenoids may increase the activity of *Lycopersicon* fruits. Deuel *et al.* (2) in their work on the stereochemical configuration and provitamin A activity report that the lycopene from tangerine tomatoes was inactive in doses of 60 mgm. Ellis and Hamner (3) have shown that there is a greater concentration of carotene in the stem end of ripe tomato fruit than in the blossom end, while the supply of mineral nutrients produced only slight variations in the carotene content of the product.

In view of the claims made for the vitamin A activity of commercially produced tomato juice, it appeared of interest to determine how well these claims were substantiated by biological assay. An opportunity was also

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Contribution from Food and Drugs Laboratory, Department of National Health and Welfare, Ottawa, Canada.

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[3] *Laboratory Assistant.*

afforded through the co-operation of Mr. M. B. Davis of the Division of Horticulture, Department of Agriculture, Central Experimental Farm, Ottawa, to determine the vitamin A activity of tomato juice produced from different varieties of tomatoes.

Sampling and Methods

Commercial samples of the known brands offered for sale in the province of Ontario were purchased from retail grocery stores during June, 1945. Each sample consisted of three cans (20 fluid oz.) each of the same code number. The samples of tomato juice were prepared at the Fruit and Vegetable Products Laboratory of the Division of Horticulture, Central Experimental Farm, Ottawa, under the supervision of Dr. Mary MacArthur, from different varieties of tomatoes, which were grown and picked under the supervision of Mr. Wm. Ferguson at the Division of Horticulture, Central Experimental Farm, Ottawa. These samples consisted of three cans (20 fluid oz.) each prepared from tomatoes gathered and processed during August, 1945.

The vitamin A activity was determined biologically by the vaginal smear method (7) using the (1934) International Standard B Carotene in cocoanut oil as reference standard. The tomato juice was administered by stomach tube and dilutions for the doses were made with water. The carotene was determined by the method recommended by the A.O.A.C. (1) for tomato products using the Evelyn photoelectric colorimeter and B carotene as the standard. The carotene as determined by this method using magnesium carbonate as the adsorbent is usually termed "crude carotene".

Results

It has been shown by Harris *et al.* (5) and confirmed by Pugsley *et al.* (7) that tocopherol improves the utilization of vitamin A and carotene, apparently by protecting these substances against oxidation in the gastrointestinal tract. It was suggested by Pugsley *et al.* (7) that in products where the addition of tocopherol was suspected this factor could be eliminated in biological assays by feeding it with the doses of the standard and sample since maximum responses were shown at dosage levels of 0.5 mgm. Oser and Melnick (6) in their work on the heat processed vegetables added tocopherol in order to eliminate this factor.

In order to determine whether there was a substance in tomato juice showing the tocopherol effect, assays were carried out on a sample of laboratory processed tomato juice with the addition of α-tocopherol (0.5 mgm. with the doses) and without tocopherol against the International Standard Carotene without tocopherol and with 0.5 mgm. The results are shown in Table I. It is seen that no significant enhancement of the vitamin A activity of the tomato juice was obtained by the addition of the tocopherol (Assays 1 and 2). On the other hand when tocopherol was added to both standard and sample (Assay 3) the vitamin A activity of the tomato juice tends to be decreased

TABLE I

THE EFFECT OF THE ADDITION OF α-TOCOPHEROL ON THE VITAMIN A ACTIVITY OF TOMATO JUICE

Assay		Ratio, sample / standard	Limits of error, % Sm* × 1.96	Potency in I.U. per cc.	Range of potency, I.U. per cc.
1	Tomato juice vs. intern. standard	0.66	83.6–119.5	8.25	6.90– 9.85
2	Tomato juice + α-tocopherol vs. intern. standard	0.68	79.5–125.8	8.47	6.73– 10.65
3	Tomato juice + α-tocopherol vs. intern. standard + α-tocopherol	0.57	82.4–121.4	7.15	5.90– 8.70
4	Intern. standard vs. intern. standard + α-tocopherol	1.17	84.8–117.8	117.2	99.5–138.0

Sm = standard error of assay.

TABLE II

THE VITAMIN A POTENCY AND CAROTENE CONTENT OF TOMATO JUICE

	Vitamin A, I.U. per cc.	Range of vitamin A values in I.U. per cc., Sm×1.96	Carotene, γ per cc.	Ratio, vitamin / carotene
Brand				
A	6.5	4.7– 8.9	5.0	
B	7.8	6.0–10.0	6.0	
C	7.8	6.4– 9.7	5.2	
D	9.1	8.0–10.3	5.9	
E	10.6	8.7–12.9	6.6	
F	10.8	9.4–12.3	5.8	
G	11.5	10.0–13.2	6.2	
H	12.4	9.6–16.0	5.2	
I	14.5	11.5–18.6	6.0	
J	14.6	11.1–19.2	5.0	
K	14.6	10.7–19.9	5.0	
L	14.9	12.2–18.2	6.4	
M	15.3	11.9–18.7	4.4	
N	15.6	13.3–18.2	6.6	
Mean	11.6	11.0–12.2	5.7 ± 0.22	
Variety				
Bonny Best	7.0	5.4– 9.0	4.7	1.49
Earliana	7.6	6.2– 9.3	4.2	1.81
John Baer	8.6	7.0–10.7	5.4	1.59
Harkness	9.1	7.5–11.0	4.4	2.07
Sugawara	9.5	8.1–11.1	6.6	1.44
Rutgers	9.8	8.2–11.6	5.6	1.75
Clark's Early	13.6	11.2–16.4	5.2	2.61
Bounty	15.6	13.2–18.5	7.2	2.17
Mean	10.4	8.7–11.1	5.4 ± 0.33	1.87 ± 0.12

owing to the enhancement of the vitamin A activity of the standard. Confirmation of the enhancement of the standard is shown in Assay 4 where the standard is assayed against itself. These results indicate there is a substance in tomato juice showing the properties of α-tocopherol and it was considered that a more accurate evaluation of the vitamin A activity of this product could be obtained without the addition of tocopherol, since the substance is inherent in the product.

Table II shows the vitamin A activity and the carotene content of the different commercial brands and of the different varieties examined. The vitamin A values of the commercial brands show a minimum of 6.5 I.U. per cc., a maximum of 15.6 I.U. per cc., and a weighted mean of 11.6 I.U. per cc., with a range of 11.0 to 12.2 I.U. per cc. The carotene values of the commercial brands show a minimum of 4.4 γ per cc., a maximum of 6.6 γ per cc., and a mean of 5.7 γ per cc., with a standard deviation of 0.22. It was not feasible to do the assays for vitamin A and the carotene determinations on the same can of tomato juice. Although cans of the same code number of the commercial samples were purchased it is quite possible that these did not represent the same batch of tomatoes and hence the values are not necessarily directly comparable. However, in the case of the samples from the different varieties, the three cans obtained represented the juice from the same batch of tomatoes and were prepared under the same conditions. In this case the vitamin A activity was determined on one can and the carotene on another and the values should be directly comparable. The values obtained for the juice produced under these experimental conditions are within the same range as those for the commercial brands. The values for the Bounty and Clark's Early varieties are somewhat higher than the others, but it is considered that further samples should be examined under varying conditions before any definite statements can be made as to the significance of the values for a specific variety. The correlation between the vitamin A activity and the carotene content of the different varieties was calculated and the coefficient $r = +0.667$ signifies that a possible correlation exists between these values. With p at 0.1, r at six degrees of freedom equals 0.621 and with p at 0.05, r at six degrees of freedom equals 0.706.

In the determination of carotene it was considered of interest to compare the values calculated as carotene on the petroleum benzene extract (after washing with 90% methyl alcohol) with those obtained after adsorption of the lycopene-like compounds on magnesium carbonate. The results are shown in Table III. It is seen that the carotene values are considerably reduced after adsorption of the lycopene-like compounds. The ratios of the values obtained before adsorption to those obtained after adsorption show considerable variation, but tend to be higher in the commercial brands than for the different varieties. The significance of these results is unknown, but undoubtedly it is related both to ripeness and variety of the tomatoes.

TABLE III

THE CAROTENE VALUES BEFORE AND AFTER ADSORPTION ON MAGNESIUM CARBONATE

	Carotene, γ per cc. after adsorption	Carotene, γ per cc. before adsorption	Ratio, $\frac{\text{before adsorption}}{\text{after adsorption}}$
Brand			
M	4.4	48	10.9
K	5.0	54	10.8
A	5.0	59	11.8
J	5.0	69	13.9
C	5.2	37	7.1
H	5.2	82	15.8
F	5.8	82	14.1
D	5.9	79	13.4
I	6.0	60	10.0
B	6.0	40	6.7
G	6.2	44	7.1
L	6.4	65	10.1
N	6.6	79	12.0
E	6.6	81	12.3
Variety			
Earliana	4.2	31	7.4
Harkness	4.4	44	10.0
Bonny Best	4.7	27	5.7
Clark's Early	5.2	37	7.1
John Baer	5.4	36	6.7
Rutgers	5.6	44	7.8
Sugawara	6.6	42	6.4
Bounty	7.2	35	4.9

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CLOSTRIDIUM BOTULINUM TYPE A TOXOIDS[1]

By Christine E. Rice,[2] E. F. Pallister,[3] L. C. Smith,[3] and G. B. Reed[4]

Abstract

Highly toxic filtrates of cultures of *C. botulinum*, Type A, when treated with 0.5% formalin and incubated at 37° C. for several weeks became non-toxic. These toxoids induced resistance in mice and guinea-pigs to several thousand times the amount of Type A botulinus toxin required to kill a normal animal of the same species.

In general, three 1.0 ml. doses of the fluid toxoid protected over 60% of inoculated mice against 100,000 lethal doses of Type A botulinus toxin. Six weeks after receiving a 5.0 ml. dose of fluid toxoid, some 88% of guinea-pigs survived the injection of 160,000 lethal doses of Type A toxins. The antitoxic titres of their sera ranged from < 0.001 to 10 units per ml. Alum precipitation increased the effectiveness of the toxoid as an immunizing agent in mice; two 1.0 ml. doses protected about 80% of mice against 100,000 lethal doses of Type A toxin. In guinea-pigs, the immunity induced by 5.0 ml. of alum-precipitated toxoid was similar to that observed following the injection of 5.0 ml. of the fluid material. No correlation was apparent between the flocculative titre of the culture filtrates before and after formalinization and their antigenicity.

Since Weinberg and Goy (13, 14), in 1924, demonstrated that botulinus toxin, like many other bacterial toxins, may be detoxified by formalin, a number of investigators have prepared toxoids from Types A, B, C, and D botulinus toxin (2-6, 8, 11, 12, 15). Amounts of formalin ranging from 0.3 to 1.0% have been employed. These toxoids, shown to confer a high degree of protection on guinea-pigs (15), have been used in the immunization of cows, sheep, and chickens (1, 3, 8) and in horses in the production of botulinus antitoxin (12). Their application in human immunization has also been reported (11). Although these investigations have been on the whole rather limited in extent, they have shown that botulinus toxin may be readily detoxified and that the resultant product has considerable antigenicity. This paper outlines procedures used in the preparation of experimental quantities of Type A toxoid of high antigenic value.

Methods

Preparation and Testing of Toxin

Type A botulinus toxoid was prepared from cultures of *C. botulinum*, Type A, Strain A Hall, grown on a medium consisting of a tryptic digest of casein, yeast extract, and glucose (9, 10). After four to five days' incubation when autolysis of the organisms was approximately complete, the cultures were filtered through paper pulp and Mandler candles. The pooled filtrates were tested for sterility, toxicity, and combining activity with botulinus antitoxin.

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Test of Toxicity

Serial, twofold dilutions of toxin were made in buffered gelatine solution* and 1.0 ml. amounts injected intraperitoneally in mice weighing approximately 20 gm.; if heavier or lighter the dosage was adjusted accordingly. When available, five mice were used for each dilution. All deaths with typical symptoms of botulinus toxemia that occurred within four days were recorded. Toxin titres were expressed in LD_{50}, calculated by the method of Reed and Muench (7). For the titration of toxicity for guinea-pigs, animals weighing 200 to 300 gm. were used. Injections were made subcutaneously.

Tests of Combining Activity with Botulinus Antitoxin

A divalent botulinus antitoxin, Lederle No. 144*H*43*J*, adopted as an arbitrary 'standard' at the unit titre stated on the label, was used in determining both the L+/10 and Lf dose of the filtrates.

Because of the scarcity of guinea-pigs, mice were employed in titration of the L+/10 dose of individual toxins. This amount, the L+/10 dose, was considered arbitrarily as the least amount of toxin that, when mixed with 0.1 unit of the 'standard' antitoxin, would kill a 20 gm. mouse within four days with characteristic symptoms of botulinus toxemia; most deaths occurred within 2 to 17 hr. The mixtures of diluted toxin and antitoxin were allowed to stand for one hour at room temperature before injection.

To determine the flocculative activity of the toxin, 2.0 ml. amounts of diluted toxin were added to varying amounts of the 'standard' antitoxin. After shaking, the mixtures were incubated at 40° C. and observed carefully till flocculation occurred. Whereas, in 1:1000 dilution, purified acid-precipitated botulinus toxins usually exhibited flocculation in 10 to 20 min., the crude culture filtrates frequently required two to four hours.

Preparation of Toxoid

Fluid Toxoid

Percentages of formalin from 0.3 to 1.0% were added to the filtered, sterile toxins. After thorough shaking the material was incubated for various periods at 37° C. The containers were rotated once daily to slightly mix the fluid. Samples were removed at suitable intervals and tested for toxicity in mice (1.0 ml.) and guinea-pigs (5.0 ml.).

Alum Toxoid

To the fluid toxoid, a sufficient amount of a sterile 10% aluminium potassium sulphate solution was added, with constant stirring, to give a final concentration of about 1.25%. The precipitate was allowed to settle overnight, the supernatant siphoned off, the volume restored to the original with sterile salt solution, and the precipitate again allowed to settle. Two similar washings were carried out and the final precipitate made up to the original

* *Gelatine*	*2 gm.*
Disodium phosphate	*10 gm.*
Water	*1000 ml.*

volume of the fluid toxoid with saline. That this precipitate contained the greater part of the antigenic material was shown by the following experiment.

To the first supernatant siphoned from the initial precipitate from one lot of toxoid, the same amount of alum was added as originally. A further precipitate formed, in bulk, approximately half that of the first precipitate. The second precipitate was washed in the same manner as the first. When tested for antigenicity in guinea-pigs and mice, the first precipitate showed good immunizing properties whereas the second afforded no significant protection to either animal species.

A few lots precipitated with calcium phosphate did not appear as potent antigens as the alum-precipitated lots.

Immunizing Value of Toxoids

The immunizing activity of each lot of fluid and alum toxoid was titrated in mice and guinea-pigs. The immunity level in these injected animals was ascertained by determining their resistance to multiple lethal doses of 'standard' Type A toxin, No. 29, a dried, purified product prepared by the method of Stevenson, Helson, and Reed (9, 10). The LD_{50} of this toxin for a 250 gm. guinea-pig was approximately 0.05 μgm.; for a 20 gm. mouse, approximately 0.001 μgm.

Mice

Two or three 1.0 ml. doses of toxoid were injected intraperitoneally or subcutaneously at intervals of seven days. Ten days after the last dose the mice were divided into two lots; one lot was given 10,000 LD_{50}, the second 100,000 LD_{50} of the 'standard' Type A toxin intraperitoneally. The animals were observed closely for 10 days for symptoms of botulinus toxemia.

Guinea-pigs

The guinea-pigs used in testing for residual toxicity in each toxoid preparation were also used to determine the relative antigenicity of these products. Six weeks after the injection of 5.0 ml. or more of toxoid they were bled and two days later given intraperitoneally a challenging dose of 'standard' Type A toxin of approximately 160,000 to 480,000 m.l.d., and kept under close observation for three weeks. Their sera were titrated for antitoxic potency in mice.

For the determination of antitoxic titre, 'standard' Type A toxin, No. 29, diluted to contain the L+/10, L+/100, and L+/1000 dose in 0.5 ml., was mixed with equivalent amounts of serial dilutions of test serum. The L+/10 dose of the 'standard' toxin for a 20 gm. mouse was approximately 15.6 μgm. After standing for one hour at room temperature, 1.0 ml. of each mixture was injected into 20-gm. mice; where possible three to five mice were used for each mixture. In the controls, 0.1, 0.01, and 0.001 units of 'standard' antitoxin were added to the L+/10, L+/100, and L+/1000 amount of toxin respectively. The animals were observed for four days.

Results

Toxicity and Combining Activity of Filtrates

All of the culture filtrates used in preparation of the Type A toxoids were of moderately high potency containing from 25,000 to 800,000 LD_{50} for the mouse per ml.; a 50 to 75% loss in toxicity occurs during filtration of the autolysed whole culture. The Lf values for these filtrates ranged from 1.5 to 7.1. As Table I illustrates, there was some general relationship between the toxicity of these filtrates and their flocculative activity with 'standard' antitoxin. The L+/10 dose for 20-gm. mice, determined for only six of the filtrates, ranged from 0.125 to 0.66 ml., approximately 25,000 to 50,000 m.l.d.; no definite correlation was noted between the L+/10 and the Lf dose of these preparations. The Lf dose of the 'standard' toxin, No. 29, was 0.141 mgm., the L+/10 dose, as stated above was 0.0156 mgm., which represents a considerably closer relationship between the two values than was observed with the culture filtrates.

TABLE I

RELATIONSHIP BETWEEN THE TOXICITY OF *C. botulinus* A TYPE TOXINS FOR MICE AND THEIR FLOCCULATIVE ACTIVITY WITH THE 'STANDARD' ANTITOXIN (LEDERLE NO. 144*H*163*K*)

Number of pooled filtrates tested	Toxicity for mice, LD_{50}/ml.	Lf	
		Mean value	Standard deviation
5	25,000	1.6	0.14
9	50,000	3.4	0.75
14	100,000	4.0	0.67
7	200,000	5.9	0.64
3	400,000	5.3	1.31
2	800,000	6.4	—

Detoxification with Different Amounts of Formalin

The first lot of toxin treated with 1.0% formalin proved to be a poor antigen. Mice given three 1.0 ml. doses failed to survive the injection of 50 to 100 m.l.d. of Type A toxin. Accordingly, the second lot of pooled culture filtrate was divided into four 500 ml. portions to which 0.3, 0.4, 0.6, and 0.8% formalin was added respectively. Detoxification was effected in two days with 0.8% formalin, in five days with 0.6%, and in six days with 0.3%. The lots treated with 0.3, 0.4, and 0.6% formalin produced a higher level of immunity in mice than that to which 0.8% formalin had been added. For subsequent batches of toxin, 0.5% formalin was used, which appeared sufficient to detoxify the most potent toxins included in this series. With larger amounts of toxin, 8 to 20 litres, it seemed advisable to extend the periods of incubation to three or four weeks, although some lots appeared detoxified in less than two weeks. Of some 22 large lots of toxoid treated in this manner, 21 produced no symptoms of toxemia in mice or guinea-pigs, one

lot induced small areas of necrosis at the site of injection but no other discernible effect. The flocculative rate of the fluid toxins with botulinus antitoxin was much slower than that of the toxoids from which they had been prepared; the Lf was usually decreased.

Immunizing Value of Fluid and Alum Toxoids

In general, toxoids that appeared of high immunizing potency in mice were also very effective in guinea-pigs; some slight disagreement was noted with a few individual preparations.

In Mice

Table II summarizes results of tests of the immunizing value of two lots of fluid and alum toxoids in mice. It will be noted that after three doses of fluid toxoid, the majority, 75 to 87%, of mice tested survived 10,000 LD_{50} of

TABLE II

DEGREE OF RESISTANCE IN MICE INJECTED WITH FLUID AND ALUM TYPE A TOXOIDS

Lot number	State of toxoid	No. of immunizing doses	Challenging dose of 'standard' Type A toxin No. 29			
			100,000 LD_{50}		10,000 LD_{50}	
			No. tested	Survival, %	No. tested	Survival, %
9	Fluid	3	58	69	55	87
11	Fluid	2	120	21	122	32
9	Alum	1	56	48	53	54
9	Alum	2	52	80	60	87
11	Alum	1	93	41	112	57
11	Alum	2	179	81	163	82

the 'standard' Type A toxin; a smaller, but still significant proportion, 50 to 69%, withstood 100,000 LD_{50}. Two doses of fluid toxoid were definitely less effective than three, only 32% being protected against 10,000 LD_{50}, 21% against 100,000 LD_{50}. One dose of fluid toxoid conferred no detectable immunity in mice. One dose of alum toxoid, on the other hand, induced a higher degree of immunity than two doses of fluid toxoid although slightly less than three doses of the latter (Table II). After two injections of alum toxoid the proportion of mice resistant to Type A toxin was approximately the same as in the group that had received three doses of fluid toxoid. The alum-precipitated product appeared therefore to be the better immunizing agent in mice.

In Guinea-pigs

It is apparent from Table III, however, that a single large dose, 5 ml. or more, depending upon the weight of the animal, of fluid and alum toxoid had about the same protective effect in guinea-pigs. In each case the resistance developed was very high, 77 to 79% respectively of the animals surviving the

TABLE III

RESISTANCE TO 'STANDARD' TYPE A BOTULINUS TOXIN OF GUINEA-PIGS SIX WEEKS AFTER INJECTION OF ONE DOSE OF FLUID OR ALUM-PRECIPITATED TYPE A BOTULINUS TOXOID

State of toxoids	Challenging dose of 'standard' Type A toxin No. 29					
	160,000 m.l.d.*		320,000 m.l.d.*		480,000 m.l.d.*	
	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %
Fluid	149	88	46	78	39	77
Alum	99	89	45	82	34	79

** Minimum amount of 'standard' toxin No. 29 required to kill a 200 to 300 gm. guinea-pig within 10 days.*

injection of 480,000 times the amount of 'standard' Type A toxin that killed normal 200 to 300 gm. guinea-pigs within 10 days.

Sera taken from these guinea-pigs six weeks after injection of fluid or alum toxoid varied in titre from <0.001 to 10 units per ml.; the majority, 56 and 55% respectively, had titres between 0.001 and 1.0 units per ml. (Table IV).

TABLE IV

TYPE A ANTITOXIC TITRE OF SERA OF GUINEA-PIGS SIX WEEKS AFTER INJECTION WITH ONE DOSE OF FLUID OR ALUM-PRECIPITATED TYPE A BOTULINUS TOXOID

State of toxoid	No. of sera tested	Percentage with titres of:				
		<0.001 units/ml.	0.001 to 0.01 units/ml.	>0.01 to 0.1 units/ml.	>0.1 to 1.0 units/ml.	>1.0 to 10 units/ml.
Fluid	188	11	13	20	36	20
Alum-precipitated	173	14	11	18	37	20

When these data were analysed it appeared that most of the guinea-pigs, whose sera had an antitoxic titre of over 0.01 units per ml., survived a challenging dose of 160,000 to 480,000 m.l.d. of Type A toxin, whereas with a titre of less than 0.001 units per ml. their chances of survival were considerably less (Table V).

No relationship was apparent between the Lf values of the toxins and toxoids prepared from them and the antigenicity of the latter. Some toxoids of high immunizing value were derived from toxin of low Lf titre, and conversely, some toxoids of relatively low immunizing value were prepared from toxins of high Lf titre (Table VI). The Lf value of a Type A botulinus culture filtrate did not seem, therefore, to provide a reliable criterion of its probable antigenic potency as a toxoid.

TABLE V

RELATIONSHIP BETWEEN ANTITOXIC TITRES AND RESISTANCE OF GUINEA-PIGS TO MASSIVE DOSES OF BOTULINUS TYPE A TOXIN, No. 29

Antitoxic titre, units/ml.	Challenging dose of toxin						Total no. tested	Survival, %
	160,000 m.l.d.		320,000 m.l.d.		480,000 m.l.d.			
	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %		
<0.001	22	45	14	43	10	70	46	50
>0.001 <0.01	18	72	8	95	7	71	33	74
>0.01 <0.1	51	90	11	91	5	80	67	87
>0.1 <1.0	90	98	17	88	21	90	128	95
>1.0 <10.0	31	100	20	95	8	88	59	94

TABLE VI

COMPARISON OF THE LF VALUES OF TOXIN AND FLUID TOXOID WITH THE IMMUNIZING ACTIVITIES IN MICE AND GUINEA-PIGS OF ALUM-PRECIPITATED TOXOID

Toxoid lot No.	Lf, toxin	Lf, toxoid	Protection in mice*		Protection in guinea-pigs**	
			No. injected	% Protected	No. injected	% Protected
10	2.0	—	10	100	3	100
14	2.0	1.8	10	70	7	100
12	2–3	2.8	23	51	5	100
13	2–3	2.2	8	88	7	100
21	2–3	2.2	25	86	5	100
23	2–3	2.9	17	89	5	100
9	3–4	—	14	83	10	100
17	3–4	1.8	23	52	2	100
29	3–4	3.8	16	100	7	86
20	4–5	4.7	32	87	4	75
30	4–5	4.4	16	81	10	50
7	5–6	—	14	92	9	100
18	5–6	2.8	10	100	9	100
19	5–6	2.8	19	79	7	100
22	5–6	2.9	25	93	5	100
26	5–6	4.4	31	86	10	100
27	5–6	4.1	35	91	11	75
24	6–7	4.1	65	83	10	78
25	6–7	4.6	37	94	11	80
28	7–8	4.4	13	90	5	40
15	7–8	3.5	17	94	8	100
16	7–8 8–9	7.1	13	88	8	100

** Percentage of mice resistant to 10,000 and 100,000 LD_{50} of Type A botulinus toxin after injection of two 1.0 ml. doses of alum-precipitated toxoid.*

*** Percentage of guinea-pigs resistant to 160,000 m.l.d. of Type A toxin six weeks after injection of 5.0 ml. of Type A alum-precipitated toxoid.*

Stability of Alum-precipitated Toxoid

Samples of three lots of alum-precipitated toxoid were stored in 20 ml. bottles at 4° to 6° C., at room temperature and at 37° C. Six and nine months later the antigenic activity of each lot was determined in mice and guinea-pigs and compared with the activity of freshly prepared toxoids. Samples stored at 37° C. had lost much of their immunizing activity. Samples stored in the cold room and at room temperature protected about the same proportion of mice as originally. The antitoxic titres of sera of guinea-pigs injected with these stored materials fell within the range noted in Table III for freshly prepared toxoids.

Discussion

These data indicate that it is possible to prepare toxoids of high potency in mice and guinea-pigs by treatment of culture of *C. botulinum* Type A with formalin.* Furthermore these toxoids appear relatively stable under ordinary conditions of storage. When the supply of guinea-pigs is limited, mice appear to be satisfactory test animals both in titrating the toxicity of the filtrates and the immunizing properties of the material and also in determining the antitoxic titre of sera from immunized guinea-pigs.

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** Since this paper was completed, Hottle and Abrams (Hottle, C. A. and Abrams, A. J. Immunol. 55 : 183-187. 1947) have made the highly significant observation that crystalline botulinum Type A toxin may be detoxified with formalin and that the resulting toxoid is an efficient antigen. As their method of determining antigenicity is very different from the one described in this paper it is not possible at present to compare the animal protective value of toxoids prepared from pure toxin and unpurified culture filtrates.*

CLOSTRIDIUM BOTULINUM TYPE B TOXOIDS[1]

By Christine E. Rice,[2] L. C. Smith,[3] E. F. Pallister,[3] and G. B. Reed[4]

Abstract

Fluid and alum-precipitated *C. botulinum* Type B toxoids were prepared by methods very similar to those used in the production of Type A toxoid, as described in a preceding paper. These Type B toxoids had little protective effect in mice but induced a moderately high degree of immunity in guinea-pigs as shown by their resistance to multiple lethal doses of Type B toxin and the development of Type B antitoxin. A relationship was observed between the Type B antitoxic titre and resistance to Type B toxin.

Methods of preparing *Clostridium botulinum* Type A toxoid have been outlined and their properties described in a previous publication from this laboratory (1). The present paper discusses the results obtained when these methods were applied with minor modifications in the production of *C. botulinum* Type B toxoid. The Type B strain, No. 7949, used in toxin production, has been maintained in our collection for a number of years since it was received from the National Type Culture Collection. It came originally from the National Canner's Association collection where it was listed as No. 213B.

Experimental Methods

The Type B cultures were grown on the casein digest medium mentioned above for four to five days, a somewhat shorter period than the six to seven days used in Type A toxin production (1). Maximum lysis of the culture was usually apparent at this time; if incubated for a longer period a decline in toxicity and flocculation titre occurred. After filtration through paper pulp and Mandler candles, the Type B culture filtrates were tested for sterility and for toxicity in mice and guinea-pigs. The sterile filtrates were treated with varying percentages of formalin, usually 0.6% and incubated until detoxified for guinea-pigs. The antigenicity of the detoxified material was tested in mice and guinea-pigs. Fluid toxoid was precipitated with alum by the procedure previously described for Type A toxoid.

Results

Relative Toxicity of Type B Toxin for Mice and Guinea-pigs

As shown by Stevenson, Helson, and Reed (2) there is a marked difference in the sensitivity of guinea-pigs and mice to Type B toxin. In the case of a representative sample of Type B filtrate, 0.00002 ml. killed a 480 gm. guinea-pig within four days, while the killing dose for a 20 gm. mouse in the

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same interval was 0.0017 ml., that is, 1.0 ml. of this toxin represented 50,000 m.l.d. for the guinea-pig but only 588 m.l.d. for the mouse. Six other Type B filtrates contained 600 to 1400 mouse m.l.d. per ml. In contrast, Type A culture filtrates had approximately the same degree of toxicity per kilogram of body weight in mice and guinea-pigs. Even more striking differences in the relative toxicity for mice and guinea-pigs were noted with the dried acid-precipitated Type B toxin, No. 135, selected as 'standard' for testing the tolerance of immunized animals and in determining the antitoxic content of their sera. The minimum lethal dose of this toxin for a 20 gm. mouse was approximately 2.5 μgm., for a 250 to 300 gm. guinea-pig approximately 0.005 μgm. A previously described (2) Type A precipitated toxin was some 2500 times more toxic for mice than this Type B precipitated toxin but the Type A and Type B toxins had approximately the same toxicity for guinea-pigs.

Combining Activity of Toxin with Antitoxin

Since a standardized univalent Type B antitoxin was not available, a divalent botulinus antitoxin containing a specified unit titre of Type A and Type B antitoxin (Lederle purified globulin preparation No. 144*H*163*K*) was used in determining the combining activity of Type B toxin with antitoxin. This procedure seemed justifiable in that Type A and Type B botulinus toxins appeared to be unrelated antigenically as shown, both by the fact that univalent Type A antitoxin had no significant neutralizing activity for Type B toxin and that mice and guinea-pigs immunized with Type A or Type B toxoids exhibited no reciprocal immunity.

In the *in vitro* tests of combining activity with antitoxin, Lf values ranging from 2.3 to 7.3 were obtained for seven lots of toxoid; double zones of flocculation were encountered with two of these lots. No correlation was noted between Lf values and toxicity for mice.

The combining activity of the Type B filtrate per unit of antitoxin as determined in mice appeared very similar to that of the Type A toxin. The L+/10 dose of the first lot of Type B filtrate was 0.125 ml., as compared with the range of 0.056 to 0.125 ml. noted for six Type A toxins. It should be noted, however, that 0.125 ml. of the Type A culture filtrates represented 25,000 to 50,000 mouse m.l.d.$_{50}$ whereas 0.125 ml. of the Type B filtrate corresponded to only 75 to 100 m.l.d. for the mouse. The L+/10 dose of the 'standard' Type B toxin No. 135, was 0.55 mgm. or approximately 220 mouse m.l.d.; that of the standard Type A toxin, No. 29, was 0.0156 mgm., approximately 15,600 mouse m.l.d.

Detoxification with Formalin

Treatment with 0.4 to 0.8% formalin at 37° C. rendered 500 ml. amounts of the first lot of Type B culture filtrate non-toxic for mice in seven days but at 10 days it was still toxic for guinea-pigs. This result emphasized the need for using guinea-pigs rather than mice in testing for residual toxicity in formalinized Type B filtrates. Formalin was added to six other larger lots

of Type B filtrate to give a concentration of 0.6%. After three weeks' incubation at 37° C., two of the six lots were still toxic for guinea-pigs. At the end of four weeks' incubation all lots were non-toxic; 200 to 300 gm. guinea-pigs given 5.0 ml. doses remained without symptoms of botulinus toxemia during a six week period of observation. The pH range of the seven Type B toxoids, 5.4 to 5.7, compared closely with that of the Type A toxoids tested, pH 5.4 to 6.0.

Antigenicity of Fluid and Alum Toxoids

Unlike the Type A toxoids, which were excellent antigens in both mice and guinea-pigs, the same amount of Type B toxoid had little immunizing effect in mice, although it was relatively efficient in guinea-pigs.

In Mice.

Table I presents the results obtained in mice with five different fluid toxoids given in three 1.0 ml. doses at weekly intervals. Seven days after the third dose the animals were tested for resistance to 5, 10, 50, and 100 m.l.d. of 'standard' Type B toxin, No. 135. Only 13% survived 5 m.l.d., 7% 10 m.l.d., none withstood 50 or 100 m.l.d. By contrast, three 1.0 ml. doses of Type A fluid toxoid protected over 60% of mice against 100,000 LD_{50} of Type A toxin, over 85% against 10,000 LD_{50} of the same.

TABLE I

RESISTANCE TO TYPE B TOXIN IN MICE IMMUNIZED WITH THREE DOSES OF FLUID OR TWO DOSES OF ALUM-PRECIPITATED TYPE B TOXOID

Number Type B toxoids tested	State	Tolerance for 'standard' Type B toxin No. 135		
		Dosage m.l.d.	No. of mice injected	Survival, %
5	Fluid	5	31	13
		10	28	7
		50	17	0
		100	10	0
5	Alum-precipitated	5	17	41
		10	13	31
		50	12	8
		100	10	10

Two doses of alum-precipitated Type B toxoid conferred on mice a somewhat higher resistance to Type B toxin than three doses of Type B fluid toxoid.

In Guinea-pigs

In testing the toxicity of the various formalin-treated Type B filtrates, 5.0 ml. doses were given to guinea-pigs weighing 200 to 300 gm.; heavier guinea-pigs received proportionately larger doses. Six weeks after injection the animals were bled and two days later tested for resistance to 100 to

10,000 m.l.d. of the 'standard' Type B toxin. A significant proportion of guinea-pigs withstood 10,000 m.l.d., which represents a much higher degree of immunity than that produced in mice (Table II). The level of resistance to homologous toxin was, however, lower than that shown in guinea-pigs receiving the same amount of Type A toxoid.

TABLE II

RESISTANCE TO TYPE B TOXIN IN GUINEA-PIGS IMMUNIZED WITH ONE DOSE OF FLUID OR ALUM-PRECIPITATED TYPE B TOXOID

Number of toxoids tested	State	Tolerance for 'standard' Type B toxin No. 135		
		Dosage	No. of guinea-pigs injected	Survival, %
5	Fluid	100	20	75
		500	10	70
		1000	42	70
		5000	12	50
		10,000	25	44
5	Alum	100	9	100
		500	2	100
		1000	16	100
		10,000	12	8

The serum of these guinea-pigs was tested for the presence of Type B antitoxin by injecting mice with mixtures of serum and varying amounts of the 'standard' Type B toxin, No. 135. Although mice were obviously less satisfactory for such titrations than the more sensitive guinea-pig, scarcity of the latter limited their use. As shown in Table III, the antitoxic titres of these guinea-pig sera ranged from <0.002 to 10 units per ml.; the majority, 63 and 66%, had titres between 0.002 and 0.1 units per ml. Of the Type-A-toxoid guinea-pigs, the majority had Type A antitoxic titres of 0.01 to 1.0 units per ml.

TABLE III

ANTITOXIN TITRES OF SERA COLLECTED FROM GUINEA-PIGS SIX WEEKS AFTER A SINGLE IMMUNIZING DOSE OF FLUID OR ALUM-PRECIPITATED TYPE B TOXOID

Number of toxoids tested	State	Number of sera tested	Percentage of guinea-pigs with antitoxic titres of units/ml.				
			<0.002	0.002 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10
5	Fluid	98	12	28	35	20	5
5	Alum	32	22	13	53	6	6

When a comparison was made between the antitoxic titres of these guinea-pigs and their resistance to Type B toxin, a fairly close relationship was observed (Table IV). The proportion of guinea-pigs protected against the

TABLE IV

RELATIONSHIP BETWEEN THE ANTITOXIC TITRE OF GUINEA-PIG SERA AND THEIR RESISTANCE TO TYPE B TOXIN

Antitoxic titre units/ml.	Challenging dose of Type B toxin No. 135							
	100 m.l.d.		1000 m.l.d.		5000 m.l.d.		10,000 m.l.d.	
	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %	No. tested	Survival, %
0.002	8	88	7	100	0	—	1	0
0.002 to 0.01	6	100	11	64	0	—	12	25
0.01 to 0.1	9	89	15	74	5	80	26	35
0.1 to 1.0	8	100	7	100	3	100	2	100
1.0 to 10	1	100	2	100	1	100	1	100
	32	94	42	81	9	89	42	31

larger amounts of toxin, 5000 and 10,000 m.l.d., was greater in the groups having higher antitoxic titres, >0.1 to 10 units. However, some of the guinea-pigs with low antitoxic titre, <0.002 unit per ml., were able to tolerate 100 to 1000 m.l.d. of Type B toxins without symptoms of toxemia.

Local Necrosis Produced by Types A and B Toxoid

Certain lots of Types A and B toxoid, both fluid and alum-precipitated, produced a slight to extensive, 6 to 10 mm. in diameter, necrotic reaction in guinea-pigs; this tendency appeared to be somewhat more marked with Type B than with Type A toxoids. Alum precipitation definitely reduced, and in some lots eliminated, these necrotizing properties. Guinea-pigs on an inadequate diet, low in vitamin C and possibly also in other essential nutritional elements, appeared to be more sensitive than normal animals, such animals injected with Type B toxoids developed much larger necrotic areas.

Discussion

From a purely immunologic standpoint, these differences in the relative toxicity and antigenicity of *C. botulinum* Type B toxins and toxoids in mice and guinea-pigs seem particularly interesting. In the mouse, the Type B culture filtrates displayed relatively low toxicity, at least as compared with that shown by Type A toxins, and the Type B toxoids derived from them were poor immunizing agents. In the guinea-pig, Type B culture filtrates were highly toxic and Type B toxoids had a very high protective potency. By contrast, the botulinus Type A culture filtrates appeared about equally toxic for mice and guinea-pigs, the Type A toxoids were highly effective in inducing resistance to Type A toxin in both animal species.

A somewhat similar situation is encountered with diphtheria and tetanus toxins in these two animal species. In the mouse, diphtheria culture filtrates exhibit only a low degree of toxicity while tetanus cultures are highly toxic;

diphtheria toxoids induce little specific immunity, tetanus toxoids are highly effective in inducing resistance to homologous toxin. In the guinea-pig, on the other hand, both diphtheria and tetanus culture filtrates are highly toxic; both toxoids induce relatively high levels of immunity.

These two instances suggest that there may be some fundamental relationship between the potential degree of reactivity of the tissues of a given animal species to bacterial toxins and the antibody response of homologous toxoids. Our knowledge of the mechanism of toxin action in the animal body is too limited to permit elaboration of these possibilities.

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A PRELIMINARY STUDY OF THE ANTIGENIC ACTIVITY OF MIXTURES OF *CLOSTRIDIUM BOTULINUM* TOXOID TYPES A AND B[1]

By Christine E. Rice[2]

Abstract

When Types A and B *Clostridium botulinum* toxoids were mixed the resultant divalent toxoid was found to have a very high protective potency against both Type A and Type B botulinus toxin. The resistance to Type A toxin induced in guinea-pigs and mice by the divalent toxoid mixture was only slightly less than that conferred by a similar volume of univalent Type A toxoid. Guinea-pigs given the divalent toxoid developed a comparable or slightly higher degree of immunity to Type B toxin than those injected with univalent Type B toxoid.

Although no evidence of reciprocal protection had been observed between Type A and Type B toxoids, admixture with Type A toxoid appreciably increased the antigenicity of the Type B toxoid for mice. Mice immunized with the divalent, A + B, toxoid exhibited a higher level of resistance to Type B toxin than those given the same number of doses of Type B toxoid alone. No adjuvant effect was noted on mixing Type A or Type B botulinus toxoids with tetanus or diphtheria toxoids.

In two preceding papers in this series (1, 2), a comparison was made of the relative antigenicity of *C. botulinum* Type A and B toxoids. This analysis showed that the botulinus Type A toxoids induced a very high level of immunity in both mice and guinea-pigs. The Type B toxoids, on the other hand, while eliciting a moderately high degree of immunity in guinea-pigs, had only a slight protective effect in mice. These differences were observed with both the fluid and alum-precipitated toxoids. No cross protection was conferred by the toxoids of either type; mice and guinea-pigs resistant to multiple lethal doses of Type A toxin died with typical symptoms of botulinus toxemia on receiving a dose of Type B toxin just sufficient to kill a normal animal of the same species. Similarly mice and guinea-pigs immunized with Type B toxoid exhibited no greater tolerance for Type A toxin than normal animals of the same species. The present paper evaluates the immunizing activities in guinea-pigs and mice of mixtures of Types A and B toxoid and compares the protection obtained with that produced by the univalent toxoids.

Relative Immunizing Activity of Type B Toxoid Alone and in the Presence of Type A Toxoid

Three lots of Type A and four lots of Type B fluid toxoids and the alum-precipitated toxoids derived from them were used in the preparation of fluid and alum-precipitated divalent, A + B, toxoids. The two types of toxoid, A and B, were mixed in equal proportions. The protective properties of the

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divalent preparations against Type A and Type B toxin were determined in mice and guinea-pigs.

In Guinea-pigs

Guinea-pigs weighing 250 to 300 gm. were injected subcutaneously with single 5.0 ml. doses of univalent Type A or Type B, or divalent, A + B, fluid and alum-precipitated botulinus toxoids. Heavier animals received proportionately larger doses. It should be noted that in the 5.0 ml. dose of divalent toxoid, the animals were receiving only 2.5 ml. of each type of toxoid, that is only half the amount given as a univalent toxoid. Six weeks after injection, the guinea-pigs were bled from the heart. Two days later they were challenged with multiple lethal doses of Type A or Type B toxins.

As had been observed with previous lots of toxoid, these univalent Type A and Type B toxoids protected guinea-pigs against several thousand times the lethal dose of homologous toxin; the resistance developed to Type A toxin was somewhat greater than to Type B toxin. The divalent, A and B, toxoids conferred a high degree of protection in these animals against both Type A and Type B toxin, Table I. The Type A antitoxic content of sera of guinea-pigs given divalent fluid toxoids, although appreciable, was on the average

TABLE I

RELATIVE RESISTANCE TO TYPE A AND TYPE B *C. botulinum* TOXIN IN GUINEA-PIGS IMMUNIZED WITH UNIVALENT TYPE A, UNIVALENT TYPE B, AND DIVALENT, A + B, TOXOIDS

Toxoid	Challenging dose of toxin					
	Type A toxin No. 29			Type B toxin No. 135		
	Dose, m.l.d.	No. of guinea-pigs injected	Survival, %	Dose, m.l.d.	No. of guinea-pigs injected	Survival, %
Fluid toxoid						
A, univalent	16,000	28	61			
	32,000	17	53			
B, univalent				100	14	79
				1000	33	67
				10,000	21	43
A + B, divalent				100	6	100
				1000	3	100
				10,000	6	100
Alum-ppt. toxoid						
A, univalent	160,000	10	70			
	320,000	8	63			
B, univalent				100	9	100
				1000	13	100
				10,000	9	11
A + B, divalent	160,000	10	80	100	14	100
	320,000	4	75	1000	19	84
				10,000	14	86

somewhat lower than that in animals injected at the same time with univalent Type A fluid toxoid. This was probably traceable, as indicated above, to the lower dosage of Type A toxoid administered in the divalent preparation. The Type A response in guinea-pigs given alum-precipitated Type A and divalent, A + B, toxoids was relatively similar.

On the other hand, although the series of guinea-pigs used was relatively smaller, the results suggested that admixture with Type A fluid toxoid slightly increased the antigenicity of the Type B toxoids for guinea-pigs. This was indicated by a somewhat higher percentage survival to multiple lethal doses of Type B toxin and by a slightly higher mean level of Type B antitoxin in guinea-pigs immunized with divalent toxoid (Table II). However, in view of the degree of variation in the antitoxic response of individual animals and the relatively small number used, these differences are of questionable significance.

TABLE II

RELATIVE ANTITOXIC TITRE OF SERA OF GUINEA-PIGS GIVEN A SINGLE DOSE OF UNIVALENT TYPE A, UNIVALENT TYPE B, OR DIVALENT A + B TOXOIDS

Toxoid	Type A antitoxic titre						Type B antitoxic titre					
	No. of sera tested	Percentage of sera with titres of (units/ml.)					No. of sera tested	Percentage of sera with titres of (units/ml.)				
		<0.001	0.001 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10		<0.001	0.001 to 0.01	>0.01 to 0.1	>0.1 to 1.0	>1.0 to 10
Fluid												
A, univalent	23	48	17	22	13	0						
B, univalent							89	13	21	49	14	2
A + B, divalent	14	57	43	0	0	0	15	20	40	13	0	27
Alum												
A, univalent	19	21	26	26	16	11						
B, univalent							27	26	15	44	7	7
A + B, divalent	52	38	21	15	21	4	67	4	5	40	39	13

In Mice

Three lots of mice were inoculated at seven day intervals with three 1.0 ml. doses of univalent or divalent fluid toxoid. Three other lots of mice received two 1.0 ml. doses at 10-day intervals of the alum-precipitated univalent or divalent toxoids. Seven days after the last dose of toxoid the Type-A-immunized animals were challenged with 10,000 to 100,000 m.l.d. of 'standard' Type A toxin No. 29; the Type-B-immunized mice with 5, 50, or 100 m.l.d. of 'standard' Type B toxin, No. 135. The mice given the divalent A + B toxoids were divided into two groups; one group was injected with 10,000 or 100,000 m.l.d. of Type A toxin, the other with 5, 50, or 100 m.l.d. of Type B toxin.

TABLE III

RELATIVE RESISTANCE TO TYPE A AND TYPE B BOTULINUS TOXIN IN MICE IMMUNIZED WITH UNIVALENT TYPE A OR TYPE B OR DIVALENT FLUID AND ALUM-PRECIPITATED TOXOIDS

Toxoid	Challenging dose of toxin					
	Type A toxin No. 29			Type B toxin No. 135		
	Dose, m.l.d.	No. of mice injected	Survival, %	Dose, m.l.d.	No. of mice injected	Survival, %
Fluid toxoids						
A, univalent	10,000	26	81			
	100,000	29	62			
B, univalent				5	53	13
				50	16	0
				100	8	0
A + B, divalent	10,000	6	67	5	23	83
	100,000	8	38	50	12	58
				100	7	71
Alum-ppt. toxoids						
A, univalent	10,000	17	100			
	100,000	16	94			
B, univalent				5	24	46
				50	11	9
				100	8	12
A + B, divalent	10,000	26	69	5	34	82
	100,000	26	62	50	40	65
				500	46	81

The results shown in Table III are in agreement with the previous observations that mice immunized with the fluid Type A toxoid are highly resistant to Type A toxin, while those injected with Type B fluid toxoid develop very little tolerance for Type B toxin (1, 2). Mice given the divalent toxoid also survived multiple lethal doses of Type A toxin although the degree of resistance was usually not as high as that following a series of doses of the univalent Type A toxoid. This again was probably referable to the fact that the mice were receiving in a 1.0 ml. dose of divalent toxoid only half as much Type A toxoid as in 1.0 ml. of the univalent preparation. By contrast, the level of immunity to Type B toxin, was higher in the mice given the divalent toxoid than in those injected with the Type B univalent toxoid alone. These differences were noted with both fluid and alum-precipitated materials. The protective potency of the alum-precipitated Type B toxoid, which was considerably greater than that of fluid preparations of the same type, was further increased by mixing with alum-precipitated Type A toxoid.

In view of the lack of reciprocal immunity observed in experimental animals injected with univalent Type A or Type B toxoids, this improvement in the antigenicity of Type B toxoid on admixture with Type A toxoid did not appear to be due to an antigenic factor common to the two types of toxoid.

Rather, it seemed as if the adjuvant property of the Type A toxoid was 'non-specific' in nature, related perhaps to some sensitizing or preparatory stimulative effect exerted by this toxoid on the antibody mechanism of the injected animal that enabled it to respond more readily to the inferior antigen, Type B toxoid. If such were the case, mixing Type B toxoid with other good immunizing agents might also increase its immunizing activity. Conversely, Type A toxoid might display a similar adjuvant effect on admixture with other less active antigens. The very limited series of experiments described below failed, however, to support either of these suggestions.

Effect of Mixing Type A or Type B Botulinus Toxoids with Other Antigens

Fluid and alum-precipitated Type A or Type B toxoids were mixed with two other bacterial toxoids, tetanus and diphtheria, and with Type 2 pneumococcus suspensions and type specific carbohydrate.

Tetanus toxoid was selected because it resembles the Type A botulinus toxoid in being prepared from a toxin highly lethal for the mouse and guinea-pig and in having good immunizing potency in both animal species.

Diphtheria toxoid resembles Type B botulinus toxoid, in being derived from culture filtrates with low toxicity for mice and high toxicity for guinea-pigs, and in inducing low immunity in mice and high immunity in guinea-pigs. The Type 2 pneumococcus suspensions and carbohydrates were chosen as examples of particulate and soluble preparations having the same type-specific antigen.

Mice were used as the principal test animals because the augmentative effect of combining Types A and B toxoids on the Type B immunity response had been more marked in these animals. They were injected at seven-day intervals with two or three 0.5 ml. doses of each of the univalent toxoids alone or with the same number of 1.0 ml. doses of the divalent mixtures. Ten days after the last injection the animals were divided into groups and tested for resistance to multiple lethal doses of homologous toxin or for protection against infection with moderately large doses of pneumococcus culture.

As shown in Table IV, the fluid tetanus toxoid used in these experiments was a poor immunizing agent in mice; its protective properties were not improved by mixing with botulinus Type A, Type B, or diphtheria fluid toxoid. Alum-precipitation greatly increased the effectiveness of this toxoid. Mixing with alum-precipitated botulinus Types A or B or diphtheria toxoids did not, however, result in any further increase in antigenic potency.

Mice injected with fluid or alum-precipitated diphtheria toxoid developed little resistance to diphtheria toxin (Table V). No improvement in their protective effect was noted when they were mixed with equal parts of Type A or Type B botulinus toxoid or with tetanus toxoid, at least in so far as could be judged from the small number of mice used.

TABLE IV

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF TETANUS TOXIN FOLLOWING IMMUNIZATION WITH TETANUS TOXOID ALONE OR IN MIXTURE WITH OTHER TOXOIDS

Toxoid	State	Dose of tetanus toxin		
		20 m.l.d.	200 m.l.d.	2000 m.l.d.
Tetanus	Fluid	1/4	0/2	—
+ botulinus Type A	Fluid	0/3	0/2	0/2
+ botulinus Type B	Fluid	0/4	0/3	—
+ diphtheria	Fluid	2/6	0/2	—
Tetanus	Alum	3/4	1/2	1/1
+ botulinus Type A	Alum	2/8	2/2	1/2
+ botulinus Type B	Alum	3/4	2/2	0/2
+ diphtheria	Alum	2/3	2/3	1/2

TABLE V

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF DIPHTHERIA TOXIN FOLLOWING IMMUNIZATION WITH DIPHTHERIA TOXOID ALONE OR IN MIXTURE WITH OTHER TOXOIDS

Toxoid	State	Dose of diphtheria toxin	
		4 m.l.d.	20 m.l.d.
Diphtheria	Fluid	2/3	2/3
+ botulinus Type A	Fluid	3/3	0/2
+ botulinus Type B	Fluid	3/5	0/5
+ tetanus	Fluid	3/3	2/2
Diphtheria	Alum	1/2	1/3
+ botulinus Type A	Alum	1/2	0/2
+ botulinus Type B	Alum	1/2	0/1
+ tetanus	Alum	1/2	0/2

It appeared from these preliminary experiments therefore that the presence of botulinus Type A toxoid had no effect on the immunizing activity of diphtheria and tetanus toxoids, nor could it be shown that the addition of either of these preparations to Type B botulinus toxoid had any such adjuvant effect as had been shown by the Type A toxoid (Table VI). Indeed the presence of diphtheria toxoid, pneumococcus vaccine, and carbohydrate seemed to depress rather than augment the antigenicity of the Type B toxoid. No indication of cross immunization with the various antigens was obtained.

Discussion

These limited data indicate that mixing *C. botulinum* Type A toxoid with Type B toxoid may improve the immunizing properties of the latter for mice and possibly to a lesser degree for guinea-pigs, notwithstanding the fact that the two antigens when used singly induce no cross protection. This effect is quite aside from that produced by alum precipitation. The Type A toxoid

TABLE VI

NUMBER OF MICE SURVIVING MULTIPLE LETHAL DOSES OF BOTULINUS TYPE B TOXIN FOLLOWING IMMUNIZATION WITH TYPE B TOXOID ALONE OR IN MIXTURE WITH OTHER BACTERIAL ANTIGENS

Antigen mixture	State	Dose of Type B toxin No. 135		
		5 m.l.d.	50 m.l.d.	500 m.l.d.
Type B botulinus toxoid	Fluid	2/5	0/5	0/2
+ Type A botulinus toxoid	Fluid	5/5	3/4	2/4
+ tetanus toxoid	Fluid	3/4	0/3	—
+ diphtheria toxoid	Fluid	0/5	0/4	0/2
+ Pneumo. Type 2 vaccine	Suspension	0/5	0/5	0/5
+ Pneumo. Type 2 SSS	Solution	0/3	0/3	0/3
Type B botulinus toxoid	Alum	3/8	1/2	1/2
+ Type A botulinus toxoid	Alum	4/4	4/4	3/3
+ tetanus toxoid	Alum	1/4	0/2	0/2
+ diphtheria toxoid	Alum	0/5	0/1	—
+ Pneumo. Type 2 vaccine	Suspension	1/7	0/3	0/4
+ Pneumo. Type 2 SSS	Solution	0/5	0/2	0/2

did not exhibit this property when mixed with tetanus and diphtheria toxoid, nor was the immunizing action of Type B toxoids in mice increased by mixing with these unrelated antigens. Since the enhancing effect of Type A toxoid was limited to a botulinus toxoid of another type, the phenomenon may not have been entirely 'non-specific' as had appeared from the first review of the situation. Some basic similarity in the chemical constitution or the physiologic effects of the two types of botulinus toxoid may have contributed in some way to this unexpected immunologic response, a similarity that was not, however, sufficiently close to call forth a definite reciprocal immunity. The problem merits further detailed investigation.

In addition to their theoretical interest, these observations also appeared of some practical importance from the standpoint of antitoxin production. If divalent botulinus toxoids are highly effective in protecting against both Type A and Type B toxin, the number of injections and the period of immunization may be reduced to about half that needed were the two univalent toxoids to be administered consecutively.

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